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Potency of Wild-Type and Temperature-Sensitive Vesicular Stomatitis Virus Matrix Protein in the Inhibition of Host-Directed Gene Expression

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The matrix (M) protein of vesicular stomatitis virus (VSV) functions in virus assembly and also appears to be involved in the inhibition of host gene expression that is a characteristic cytopathic effect of VSV infection. Previous studies have shown that expression of M protein inhibits host-directed transcription in the absence of other viral gene products and have suggested that only small amounts of M protein are required for the inhibition. In experiments described here, the potency of M protein in inhibition of host-directed gene expression was determined by cotransfecting different amounts of *in vitro*-transcribed M protein mRNA together with a target gene encoding chloramphenicol acetyl transferase (CAT) into BHK cells or PC12 cells that had been cultured in the presence or the absence of nerve growth factor. The results of these experiments showed that the potency of M protein was similar in the two cell types and was not affected by the extent of differentiation of PC12 cells. Inhibition of CAT gene expression by M protein was also independent of the nature of the promoter activating sequences of several different RNA polymerase II-dependent promoters. The amount of M protein needed to give 50% inhibition of CAT expression was estimated to be 6700–11,000 copies per cell. Earlier data that temperature-sensitive (ts) M gene mutants of VSV inhibit host transcription had been interpreted to indicate that M protein was not involved in the inhibition. When the amount of M protein expressed was taken into account, ts M protein was as effective as wild-type M protein in the inhibition of host-directed transcription at the nonpermissive temperature. Thus, inhibition of host transcription by ts M mutants of VSV is due to the potent activity of M protein, which is evident even at the low levels produced at the nonpermissive temperature.

INTRODUCTION

The matrix (M) protein of vesicular stomatitis virus (VSV) is involved in at least two separate processes in virus-infected cells, virus assembly and virus-induced cytopathology. M protein plays a major role in virus assembly by mediating envelopment of the nucleoprotein core (nucleocapsid) by the host plasma membrane during the budding process (Lenard, 1996). In addition, M protein appears to be responsible for some (though not all) of the cytopathic effects of virus infection. One of the cytopathic activities of M protein is to cause the characteristic rounding of VSV-infected cells, presumably by disrupting cytoskeletal function (Blondel *et al.,* 1990; Melki *et al.,* 1994; Ye *et al.,* 1994). M protein also has the ability to inhibit host-directed transcription and may be involved in the shutoff of host gene expression in VSV-infected cells.

M protein can inhibit host-directed transcription in transfected cells in the absence of other viral components (Black and Lyles, 1992; Paik *et al.,* 1995). Furthermore, the ability of M protein to inhibit host-directed gene expression was found to be genetically separate from its role in virus assembly (Black *et al.,* 1993). VSV infection results in pronounced inhibition of host translation as well as transcription. In several cases, defects in inhibition of host translation by VSV mutants have been correlated with mutational changes in M protein (Coulon *et al.,* 1990; Francoeur *et al.,* 1987; Stanners *et al.,* 1977), suggesting that M protein is involved in the inhibition of translation. However, expression of M protein in the absence of other viral components actually stimulates translation of cotransfected mRNAs (Black *et al.,* 1994), indicating that other viral components must be involved, perhaps together with M protein, in this aspect of VSV cytopathology. Thus, M protein expressed in the absence of other viral components affects expression of cotransfected genes at multiple levels, the overall effect of which is a marked inhibition.

The amount of M protein produced in transfected cells is usually quite small (Black and Lyles, 1992; Black *et al.,* 1993; Paik *et al.,* 1995) due to the fact that M protein inhibits its own expression from transfected DNA vectors that depend on host transcriptional activity (Black and Lyles, 1992). The amount of M protein produced in transfected cells was estimated to be about 0.1 to 0.2% of that produced in a typical virus-infected cell, suggesting that M protein has a potent effect on host gene expression.

In experiments described here, the potency of M pro-

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tein in inhibition of host-directed gene expression was determined. Quantitative approaches were used in which the amount of M protein expressed was varied in a systematic manner by transfecting different amounts of *in vitro*-transcribed M protein mRNA. The results of these experiments showed that the potency of M protein was largely independent of cell lineage or extent of differentiation as well as the nature of the promoter activating sequences of several different RNA polymerase II-dependent promoters.

The potency of M protein in inhibiting host-directed transcription provides an explanation for earlier data that implied that M protein was not responsible for inhibition of host transcription. In particular, the fact that temperature-sensitive (ts) M protein mutants inhibit host transcription at the nonpermissive temperature (Weck and Wagner, 1979a) has been interpreted to indicate that M protein is not involved in the shutoff of host transcription. In data presented here, it was shown that inhibition of host transcription by ts M mutants can now be understood in terms of the potency of M protein-induced inhibition which is evident even at the low levels of functional M protein produced at the nonpermissive temperature.

MATERIALS AND METHODS

Cells and viruses

Wild-type VSV (Indiana serotype, San Juan strain) and the ts M mutant tsM301 were grown in BHK cells as described (Lyles *et al.,* 1996). PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and 5% horse serum as described (Muller *et al.,* 1990).

Plasmids and *in vitro* transcription of mRNA

The following plasmids containing the chloramphenicol acetyl transferase (CAT) reporter gene have been described: pSV2.CAT and pRSV.CAT (Gorman *et al.,* 1982), pMLP.CAT (Chang *et al.,* 1989), pDHF(-210).CAT (Blake *et al.,* 1990), pBL.CAT2 (Angel *et al.,* 1987), and a plasmid containing a CAT gene driven by the H-2K^b promoter and 260 bp of upstream sequence (Dey *et al.,* 1992). The plasmid used for *in vitro* transcription of mRNA encoding wild-type (wt) M protein together with a 3′ poly(A) sequence has been described (Black *et al.,* 1994). The M gene of the tsM301 mutant was constructed by reverse transcription of viral RNA and polymerase chain reaction and was subcloned into the *in vitro* transcription vector as described previously (Black *et al.,* 1993, 1994). M mRNAs containing 5′ caps and 3′ poly(A) were transcribed in the presence of cap analog, 7mG(5′)ppp(5′)G, from appropriately linearized plasmid DNA by bacteriophage SP6 RNA polymerase using reagents and procedures from a commercial kit (Message Machine, Ambion, Inc.).

Cotransfection of mRNA and DNA

Protocols for cotransfection of M mRNA and CAT plasmid DNA into BHK cells (Black *et al.,* 1994) were modified to accommodate the lower levels of CAT expression in PC12 cells. Also, CAT expression in PC12 cells was found to be inhibited even by negative control RNA when higher levels of RNA were used. PC12 cells (approximately 3×10^5 cells) seeded onto polylysine-coated culture dishes were cultured in the presence of 100 ng/ml murine 7S nerve growth factor (NGF, obtained from GIBCO-BRL) as described (Muller *et al.,* 1990) for 4 days prior to transfection. Control undifferentiated PC12 cells and BHK cells were seeded 1 day prior to transfection. Cells were transfected with 2 μg of pRSV.CAT DNA together with 400 ng of RNA and 15 μg Lipofectin reagent (GIBCO-BRL) in Dulbecco's phosphate-buffered saline for 3 hr. Culture medium was replaced, and cells were incubated 24 or 48 hr prior to harvest. The total amount of RNA was held constant at 400 ng by mixing M mRNA with yeast RNA (Type XI, Sigma Chemical Co.). Transfection of BHK cells in experiments that did not involve PC12 cells was performed similarly except that 200 ng of CAT plasmid DNA, 800 ng total RNA, and 6 μg Lipofectin reagent were used. The methods for assay of CAT activity in cell extracts by acetylation of [^{14}C]chloramphenicol and conversion of percent acetylation to relative units that are linearly related to CAT activity have been described (Black *et al.,* 1993). Western blot analysis of the expression of M protein in transfected cells was performed as described (Taylor *et al.,* 1994) using anti-M protein monoclonal antibody 23H12 (Lyles *et al.,* 1988).

RESULTS

M protein-induced inhibition of gene expression is not affected by cellular differentiation

Cytopathic effects of M protein have been observed previously in several different cell types (Black and Lyles, 1992; Blondel *et al.,* 1990; Paik *et al.,* 1995; Ye *et al.,* 1994). The ability of M protein to inhibit host-directed gene expression was compared in cell types of two different lineages and extents of differentiation to determine whether cells differ in their sensitivity to M protein. This was tested by cotransfecting synthetic mRNA encoding M protein together with a target gene encoding CAT. M protein was expressed from transfected mRNA (Black *et al.,* 1994) instead of plasmid DNA to avoid the problem that M protein inhibits its own expression from DNA-based vectors that require host transcriptional activity (Black *et al.,* 1992). Expression of the CAT target gene was driven by the RSV promoter, a typical RNA polymerase II-dependent promoter that is efficiently expressed in the cells used (Muller *et al.,* 1990). The cells chosen for these experiments were BHK cells, a hamster fibroblast-like cell line commonly used for growth of VSV in

the laboratory, and PC12 cells cultured in the presence and the absence of NGF. PC12 cells are a line of rat pheochromocytoma cells that acquire many of the morphological and biochemical characteristics of differentiated sympathetic neurons when cultured in the presence of NGF (Greene and Tischler, 1976). PC12 cells were chosen for these experiments because VSV is neurotropic in rodent models of *in vivo* infection (Huneycutt *et al.,* 1994; Lundh *et al.,* 1987) and because they can be transfected with relatively high efficiency (Muller *et al.,* 1990).

BHK cells or PC12 cells cultured in the presence or the absence of NGF (approximately 3×10^5 cells per culture) were cotransfected with pRSV.CAT plasmid DNA (2 μg) together with varying amounts of mRNA encoding VSV M protein. The total amount of RNA transfected (400 ng) was held constant by mixing with yeast total RNA. Cells were harvested either 24 or 48 hr posttransfection, cell extracts were prepared, and the extracts were assayed for CAT activity by incubation with [^{14}C]-chloramphenicol in the presence of acetyl CoA. Unreacted chloramphenicol was separated from its acetylated derivatives by thin-layer chromatography, autoradiographs of which are shown in Fig. 1. CAT expression in PC12 cells was observed at 48 hr posttransfection (Fig. 1A), but was barely detectable at 24 hr posttransfection (not shown). In the experiment shown in Fig. 1A, PC12 cells cultured in the presence of NGF expressed higher levels of CAT than cells grown without NGF, although this difference was not consistently observed. Expression of M protein inhibited CAT expression in PC12 cells cultured in either the presence or the absence of NGF, but only at the highest level of transfected mRNA tested (400 ng). CAT was expressed at much higher levels in BHK cells than in PC12 cells, even at 24 hr posttransfection (Fig. 1B). When the same amount of BHK cell extract was assayed as PC12 cell extracts, nearly all of the chloramphenicol was acetylated by extracts from control transfected cells and much of it was diacetylated. This difference in CAT expression between PC12 cells and BHK cells is probably due to differences in rates of transcription and/or translation of the CAT gene, since it cannot be accounted for by differences in transfection efficiency between BHK cells and PC12 cells (Muller *et al.,* 1990, and unpublished results). M protein-induced inhibition of CAT expression in BHK cells was observed at lower levels of transfected mRNA than in PC12 cells, as shown by the lack of production of diacetylated chloramphenicol by extracts from cells transfected with 40 ng of mRNA (Fig. 1B).

The amount of M protein expressed in transfected BHK and PC12 cells was measured by Western blot analysis to determine whether the differences in M protein-induced inhibition of CAT expression in these two cell types reflected the relative levels of M protein expressed. The immunoreactivity of cell extracts was compared to

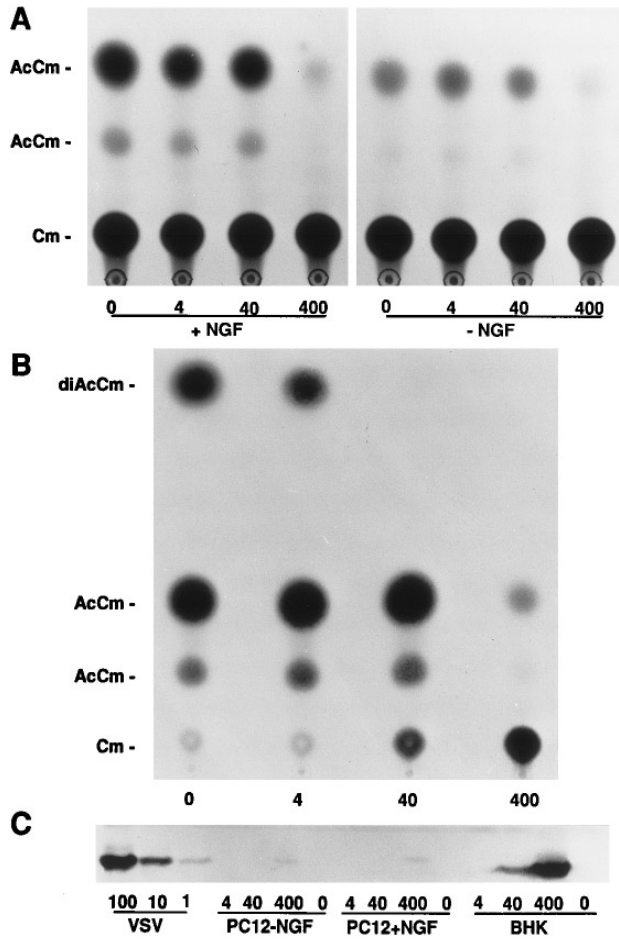

FIG. 1. Inhibition of CAT gene expression in PC12 and BHK cells by cotransfection of M mRNA. PC12 cells (approximately 3×10^5 cells per culture), cultured in the presence or the absence of NGF (A), or BHK cells (B) were cotransfected with pRSV.CAT DNA and the indicated amount of M mRNA. Cells were harvested after 48 hr (A) or 24 hr (B). CAT activity of cell extracts was determined by incubation with [^{14}C]chloramphenicol and acetyl CoA. Unreacted chloramphenicol (Cm) was separated from its monoacetylated (AcCm) and diacetylated (diAcCm) forms by thin-layer chromatography, an autoradiograph of which is shown. M protein expression in transfected cells was determined by Western blot analysis (C). Cell extracts (1/5 of the total) and the indicated amount (ng of protein) of purified VSV were subjected to SDS–PAGE, blotted onto nitrocellulose membranes, and probed with a monoclonal antibody against the VSV M protein.

that of purified virions (1–100 ng) as a standard. The data obtained at 24 hr posttransfection are shown in Fig. 1C. Similar results were obtained at 48 hr posttransfection (not shown). M protein expression was readily detected in BHK cells and was dependent on the amount of mRNA transfected. However, M protein expression in PC12 cells was clearly detected only when the cells were transfected with 400 ng of mRNA. These results suggest that the differences in inhibition of CAT gene expression between BHK cells and PC12 cells in Figs. 1A and 1B may be due to the lower level of M protein in PC12 cells.

In order to compare quantitatively the extent of M protein-induced inhibition of CAT expression in PC12 cells

TABLE 1

Correlation of Inhibition of CAT Gene Expression with Levels of Expression of M Protein in PC12 Cells and BHK Cells

Cell type	M mRNA transfected (ng/culture)	CAT activity (% of control) mean ± SEM	M protein (fraction of total × 10^6) mean ± SEM	M protein (ng/10^5 cells) mean ± SEM
PC12 + NGF	400	33 ± 8	5.0 ± 0.7	0.84 ± 0.2
PC12 − NGF	400	19 ± 2	6.3 ± 0.8	0.68 ± 0.2
BHK	4	95 ± 5	2.4 ± 0.4	0.18 ± 0.03
	40	25 ± 2	18 ± 2	1.1 ± 0.2
	400	7 ± 5	49 ± 9	3.5 ± 0.8

Note. PC12 cells (approximately 3 × 10^5 cells per culture), cultured in the presence of the absence of NGF, or BHK cells were cotransfected with pRSV.CAT DNA together with the indicated amount of M mRNA. Assay of CAT enzymatic activity and Western analysis of M protein expression were performed as in Fig. 1. CAT activity is expressed as a percentage of a negative control with no M mRNA. M protein expression is shown both on a per cell basis and as a fraction of the total cellular protein. Data shown are means ± SEM for four (PC12) or three (BHK) independent experiments.

versus BHK cells, experiments were designed to be similar to those in Fig. 1 except that extracts from BHK cells were diluted eightfold prior to analyzing CAT activity so that the extent of acetylation of chloramphenicol was linearly related to the amount of CAT enzyme. The extent of acetylation of chloramphenicol was determined by either radioanalytic scanning of TLC plates or densitometry of autoradiographs similar to those in Figs. 1A and 1B. The amount of M protein expressed was determined by densitometry of Western blots similar to that in Fig. 1C, in which the immunoreactivity of cell extracts was compared to that of known amounts of M protein from purified virions. The data from four independent experiments are shown in Table 1.

M protein-induced inhibition of CAT activity in PC12 cells cotransfected with 400 ng of M mRNA was most directly comparable to that in BHK cells transfected with 40 ng of M mRNA. CAT activity was typically 20–40% of that in control cells cotransfected with yeast RNA. The differences in activity between cells cultured in the presence versus the absence of NGF were not statistically significant. The amount of M protein expressed by PC12 cells (in either the presence or the absence of NGF) transfected with 400 ng of M mRNA was two- to threefold less than that expressed by BHK cells transfected with 40 ng of M mRNA when calculated either as a fraction of total cellular protein or on a per cell basis. Thus, PC12 cells appeared to be slightly more sensitive to the inhibitory effects of M protein. An important consideration in interpreting these data is potential differences in efficiency of transfection between PC12 cells and BHK cells. Although the transfection efficiencies of PC12 cells could not be accurately determined, the percentage of PC12 cells transfected was likely to be slightly less than the percentage of BHK cells transfected (see Discussion). If the data in Table 1 could be expressed as the amount of M protein in transfected rather than total cells, the actual level of expression would be higher than that indicated in Table 1, because the values for the amount of M protein expressed would be divided by a smaller value for the amount of total cellular protein. Since this effect would be greater for PC12 cells, this would move the data for PC12 cells even closer to those for BHK cells. Thus, the conservative interpretation of the data in Table 1 would be that there is little or no difference in the sensitivity of PC12 cells to the M protein-induced inhibition of gene expression compared to BHK cells or when cultured in the presence or the absence of NGF.

Lack of promoter specificity in M protein-induced inhibition of gene expression

Host-directed transcription in VSV-infected cells is markedly inhibited as measured by incorporation of [^{3}H]-uridine into total cellular RNA. This suggests that there is little if any promoter specificity in the virus-induced inhibition of host-directed transcription. In order to test whether this is also true of the M protein-induced inhibition of gene expression, BHK cells were cotransfected with M mRNA together with plasmid DNA encoding CAT genes under the control of different RNA polymerase II-dependent promoters (Fig. 2). The promoters chosen were the murine class I MHC (H-2K^b, closed squares) and dihydrofolate reductase (dhfr, open circles) promoters, the adenovirus major late promoter (closed circles), and the herpes simplex virus thymidine kinase promoter (open squares). These were chosen to represent promoters with a variety of upstream enhancer sequences and mechanisms of transcription initiation including traditional TATA box-containing promoters as well as a TATA box-independent promoter (dhfr). CAT activity was determined 24 hr after cotransfection of BHK cells with these CAT genes together with varying amounts of M mRNA. CAT expression from all of these promoters was inhibited to similar extents, indicating that there is little if any promoter specificity in M protein-induced inhibition of gene expression. The amount of M mRNA required to inhibit CAT expression in these experiments is less than that

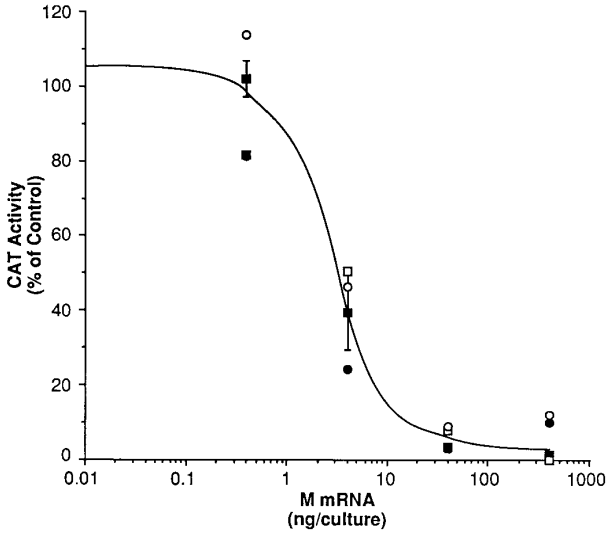

FIG. 2. M protein-induced inhibition of expression from different RNA polymerase II-dependent promoters. BHK cells (approximately 3×10^5 cells per culture) were cotransfected with the indicated amounts of M mRNA together with 0.2 μg of CAT plasmid DNA containing the following promoters: H-2K^b (closed squares), dihydrofolate reductase (open circles), adenovirus major late promoter (closed circles), and herpes simplex virus thymidine kinase (open squares). Cells were harvested 24 hr posttransfection and CAT activity was determined as in Fig. 1. Data are expressed as a percentage of a negative control with no M mRNA. Data shown are means of three independent experiments. Error bars (±SD) are shown only for data obtained with the H-2K^b promoter for clarity. Similar values were obtained for the other promoters.

needed in the experiments in Fig. 1 and Table 1. The reason for this is not known, but is probably due to the fact that in Fig. 1 the M mRNA was diluted with 10-fold more CAT plasmid DNA (2 μg) than in the experiments in Fig. 2 (0.2 μg) and in our previous study using the SV40 promoter (Black *et al.,* 1994) in order to be able to detect CAT activity in PC12 cells.

Inhibition of host-directed gene expression by temperature-sensitive M protein

The group III (M gene) ts mutants of VSV are defective in the late stages of virus assembly at the nonpermissive temperature (Lyles *et al.,* 1996; McCreedy and Lyles, 1989; Ono *et al.,* 1987). The fact that ts M protein mutants inhibit host transcription at the nonpermissive temperature (Weck and Wagner, 1979a) has been interpreted to indicate that M protein is not involved in the shutoff of host transcription. Therefore, the M protein of a typical group III mutant, tsM301, was tested for its ability to inhibit CAT gene expression. BHK cells were cotransfected with varying amounts of mRNA encoding the tsM301 M protein together with pSV2.CAT plasmid DNA and incubated for 24 hr at either the permissive (31°) or nonpermissive (39°) temperature for virus replication. Cells cotransfected with wt M mRNA served as positive controls. CAT enzymatic activity in cell extracts was assayed as in the experiments in Fig. 1. The results from four independent experiments are shown in Fig. 3.

At 31° inhibition of CAT gene expression by transfection of ts M mRNA (Fig. 3, closed squares) was slightly less than that of wt M mRNA (closed circles). Inhibition of CAT expression by wt M protein was unaffected by temperature (open and closed circles). However, inhibition of CAT expression by ts M protein at 39° (open squares) was observed only at the higher levels of transfected mRNA tested (⩾200 ng). At the lower levels of M mRNA tested, CAT expression was actually enhanced slightly in two of the four experiments.

Western blot analysis of ts and wt M protein expression is shown in Table 2. Ts M protein was expressed from transfected mRNA at 31° at levels similar to those of wt M protein. However, at 39°, expression was not detected when cells were transfected with 4 or 40 ng of ts M mRNA. Cells transfected with 400 ng of ts M mRNA at 39° expressed levels of M protein comparable to those achieved with 4 ng of wt M mRNA. Figure 4 combines the data in Fig. 3 with those in Table 2 in a logarithmic plot of CAT activity as a function of the amount of M protein expressed for both wt and ts M protein at both 31° and 39°. This plot is approximately linear in this range of CAT activities, and all of the data are close to the same line. In particular, the ts M protein was observed to be as effective in the inhibition of CAT gene expression as wt M protein even at the nonpermissive temperature (open square, indicated by an arrow).

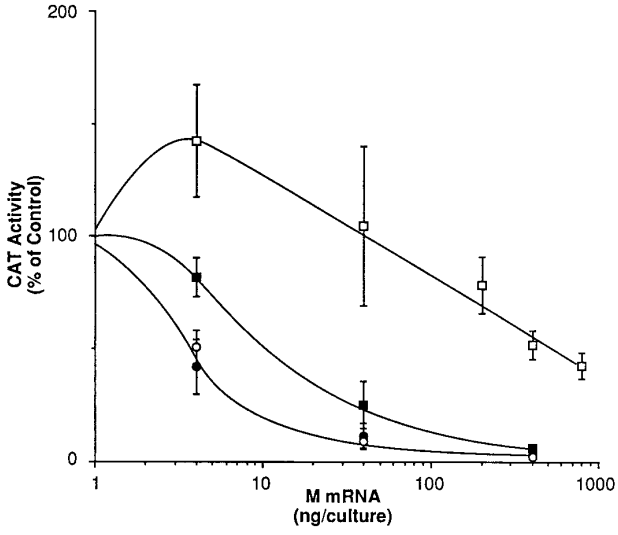

FIG. 3. Inhibition of CAT gene expression by ts M protein. BHK cells (approximately 3×10^5 cells per culture) were cotransfected with the indicated amounts mRNA encoding either ts M protein (squares) or wt M protein (circles) together with 0.2 μg of pSV2.CAT plasmid DNA. Cells were incubated for 24 hr at either 31° (closed symbols) or 39° (open symbols). CAT activity in cell extracts was determined as in Fig. 1. CAT activity is expressed as percentage of a negative control with no M mRNA. Data shown are means ± SEM of four independent experiments. Data obtained with 200 and 800 ng mRNA are from a separate series of three experiments.

TABLE 2

Western Blot Analysis of M Protein Expression in Cells Transfected with mRNA Encoding wt or tsM301 M Protein

M type	M mRNA transfected (ng/culture)	Cells incubated at 31° (ng M/ 10^5 cells)	Cells incubated at 39° (ng M/ 10^5 cells)
Wild type	400	5.5	3.5
	40	1.3	1.5
	4	0.25	0.33
M301	400	2.0	0.28
	40	0.7	n.d.
	4	0.35	n.d.

Note. BHK cells (approximately 3×10^5 cells per culture) were transfected with the indicated amounts of mRNA. Cells were incubated for 24 hr at either 31° or 39°. Levels of M protein in cell extracts were determined by Western blots as in Fig. 1 and quantitated by densitometry. Data shown are means of two independent experiments (n.d., not detected).

The amount of M protein expressed during infection of BHK cells by wt VSV and the tsM301 mutant virus was determined at varying times postinfection by Western analysis (Fig. 5) for comparison with the results obtained in transfected cells. In the case of both viruses, the level of M protein increased more rapidly at 39° (open squares and circles) than at 31° (closed squares and circles). Nonetheless, at both temperatures, M protein was expressed at levels approximately 1000-fold greater than those in transfected cells by the peak time of virus assembly from 4–6 hr postinfection. There was little difference in the levels of M protein expressed by wt VSV (circles) versus tsM301 virus (squares) even at the nonpermissive temperature. It is not known how much of the M protein in ts M mutant-infected cells is in a functional form at the nonpermissive temperature. However, the fact that it is expressed at levels that are several orders of magnitude larger than the amounts needed to inhibit host-directed gene expression provides an explanation for why these viruses are able to inhibit host RNA synthesis at the nonpermissive temperature.

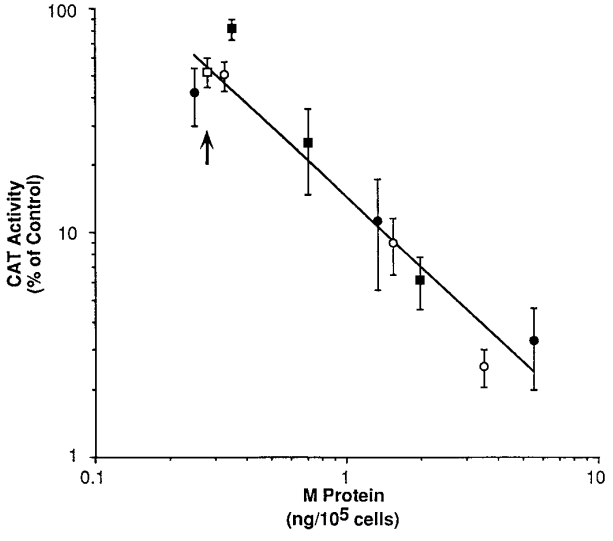

FIG. 4. Correlation of inhibition of CAT gene expression with levels of expression of wt and ts M protein. CAT activities in cells transfected with pSV2.CAT DNA from Fig. 3 are plotted as a function of the amount of M protein expressed from cotransfected M mRNA from Table 2 encoding either ts M protein (squares) or wt M protein (circles) incubated at either 31° (closed symbols) or 39° (open symbols). The arrow indicates the value obtained from transfection with 400 ng of mRNA encoding ts M protein at 39°.

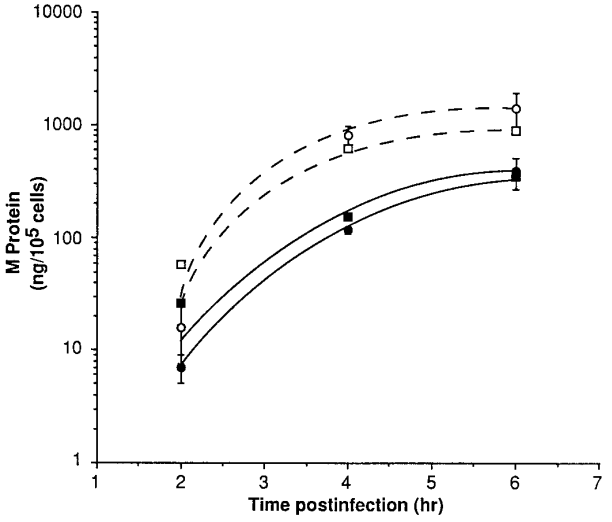

FIG. 5. Levels of M protein expressed in cells infected with wt VSV and tsM301 virus. BHK cells were infected with wt VSV (circles) or tsM301 virus (squares) for the indicated times postinfection at either 31° (closed symbols) or 39° (open symbols). Cell extracts were prepared, and the levels of M protein were determined by Western blots as in Fig. 1. Data shown are means of three independent experiments. Error bars (±SD) are shown only for data obtained with wt VSV for clarity. Similar values were obtained for data with tsM301 virus.

DISCUSSION

One of the early clues that VSV M protein might inhibit host-directed gene expression was the observation that it was difficult to express detectable amounts of M protein from plasmid vectors that depend on host transcriptional activity (Li *et al.,* 1988; Blondel *et al.,* 1990). Reasoning that M protein might be inhibiting its own expression from plasmid DNA, a cotransfection assay was used in which the M gene and the target gene encoding CAT were both under the control of the SV40 early promoter to show that M protein inhibited expression from both plasmids (Black and Lyles, 1992). It has also been shown that M protein expressed using the HIV LTR inhibits chromosomal HIV proviral expression in addition to expression from plasmid DNA when M gene expression was induced by HIV infection (Paik *et al.,* 1995). However, in all of these experiments, the level of M protein expressed was near or below the detection limit of the assays used. To circumvent the problem that M protein inhibits its own

expression from vectors that depend on host transcription, target genes have been cotransfected with M mRNA transcribed *in vitro* (Black *et al.,* 1994). This approach was used in the present study to determine the relative sensitivity of cells of different lineages to M protein-induced inhibition of gene expression.

PC12 cells appeared to be slightly more sensitive to the effects of M protein than BHK cells when the level of intracellular M protein was expressed as a fraction of the total cellular protein (Table 1). However, presenting the data in this form actually overestimates the differences between PC12 cells and BHK cells. The efficiency of transfection of PC12 cells is likely to be less than that of BHK cells, so that if the level of M protein could be expressed as a fraction of protein in *transfected* cells, this would have the effect of moving the data for PC12 cells closer to those for BHK cells. While the efficiency of BHK cell transfection with 400 ng M mRNA could readily be determined by immunofluorescence microscopy, it was difficult to determine the transfection efficiency when M protein was expressed at the lower levels seen in PC12 cells transfected with 400 ng M mRNA or BHK cells transfected with 40 ng M mRNA. Even though some of the transfected cells were clearly fluorescent, others were not clearly distinguishable from the background fluorescence (data not shown). Nonetheless, it is clear that the minor differences between PC12 cells and BHK cells in apparent sensitivity to M protein-induced inhibition of gene expression can be readily accounted for by differences in transfection efficiency and are not likely to be biologically significant.

Since no attempt was made to exhaustively survey all cell types, the data in Fig. 1 and Table 1 do not imply that all cell types will be equally sensitive to the inhibitory effects of M protein. These data do indicate that the effects of M protein are likely to be observed in mammalian cells of diverse lineages and extents of differentiation. In addition to the cell types studied here, cytopathic effects of M protein have been observed previously in human 293 cells (Black and Lyles, 1992) and HeLa-T4 cells (Paik *et al.,* 1995) and in simian COS cells (Blondel *et al.,* 1990) and CV1 cells (Ye *et al.,* 1994). Thus, the cytopathic effects of M protein are consistent with the biology of VSV, which is highly cytopathic in a wide variety of mammalian cell types. It has not been determined how far down the phylogenetic tree the cytopathic effects of M protein are observed. However, expression of VSV M protein does not affect the growth of the yeast *Saccharomyces cerevisiae* and thus is not likely to be cytopathic in yeast cells (unpublished results).

In the case of BHK cells, levels of CAT expression in cotransfected cells were inversely correlated with levels of M protein expression determined by Western blots (Table 1 and Fig. 4). The amount of M protein needed to give 50% inhibition of CAT expression was approximately 0.3–0.5 ng per 10^5 total cells (Fig. 4). From the M protein molecular weight of 26,000, this corresponds to 2000–3300 molecules per cell. The efficiency of mRNA transfection of BHK cells under these conditions has been determined to be 30–50% by immunofluorescence using mRNA for either the M protein or the VSV N protein, which is not cytopathic (data not shown). Using a conservative value of 30%, the level of M protein expression in transfected cells would thus be 6700–11,000 copies per cell. Since there are approximately 10^8 cells/ml of packed cells, this corresponds to an intracellular concentration of approximately $3–5 \times 10^{-8}$ M. This level of expression is approximately 1000-fold less than the amount of M protein in VSV-infected cells at the peak of virus assembly at 4–6 hr postinfection (Fig. 5).

The potency of M protein in the inhibition of host-directed transcription provides an explanation for earlier data, which indicated that M protein was not responsible for inhibition of host transcription in VSV-infected cells. In particular, the observation that ts M mutants of VSV inhibit host transcription at the nonpermissive temperature (Weck and Wagner, 1979a) has been interpreted to indicate that M protein is not responsible for inhibition of host transcription. These results can now be understood in terms of the potency of M protein-induced inhibition which is evident even at the low levels of functional M protein produced by the ts M mutants at the nonpermissive temperature. When the levels of expression were accounted for, the ts M protein was found to be as effective as wt M protein in inhibition of gene expression at the nonpermissive temperature (Fig. 4). The nature of the defect in ts M proteins is not completely clear. An early study attributed the defect to a more rapid turnover of the ts M protein at the nonpermissive temperature (Knipe *et al.,* 1977), while a later study suggested that the ts M protein was aggregated within infected cells (Ono *et al.,* 1987). In either case, a pool of functional ts M protein apparently exists that is capable of mediating some of the intermediate stages of virus assembly (Lyles *et al.,* 1996). While we do not know how large that pool is, the overall levels of M protein in cells infected with ts M mutants at the nonpermissive temperature are approximately 1000-fold greater than that required for 50% inhibition of CAT expression in the cotransfection experiments (compare Figs. 4 and 5).

The residual expression of small amounts of M protein could account for the results of earlier UV-inactivation experiments, which indicated that the UV target size for inhibition of host transcription was too small to be the M gene (Weck *et al.,* 1979; Dunigan *et al.,* 1986). The kinetics of UV-inactivation are complex (Dunigan *et al.,* 1986), but approximately 200- to 700-fold more UV irradiation is required to inactivate the ability of VSV to inhibit host transcription than to inactivate infectivity, leading to target size estimates of about 50 nucleotides, similar to the size of the VSV

leader RNA. The assumption in these experiments is that the ability to inhibit host gene expression is proportional to the amount of the viral component produced. However, in the case of virus-infected cells, approximately 1000-fold more M protein is produced than is necessary to shut off host transcription. The amount of UV irradiation required to reduce M protein expression by 1000-fold would be similar to that observed to be necessary to prevent inhibition of host transcription.

An overall suppression of RNA polymerase II-dependent transcription is observed in VSV-infected cells, suggesting that there is little specificity in terms of which cellular promoters are affected. Likewise, there appears to be little if any promoter specificity in the inhibition of host-directed transcription by M protein (Fig. 2). In particular, the inhibition was independent of the type of upstream enhancer sequences or the mechanism of promoter recognition (i.e., TATA-dependent versus TATA-independent promoter recognition). This result suggests that inhibition of host-directed transcription involves inactivation of some component of the basal transcription machinery. Previous studies have shown that inhibition of RNA polymerase II-dependent transcription in VSV-infected cells is at the level of transcription initiation rather than elongation (Weck and Wagner, 1979b). Based on the lack of promoter specificity and the small number of M protein molecules required, an attractive model for inhibition of host-directed transcription is that M protein either directly or indirectly inactivates one or more limiting transcription factors involved in transcription initiation. Future studies should lead to the identification of such factors.

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